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DWnt4 and Wingless Elicit Similar Cellular Responses during Imaginal Development

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***Wnt* genes encode evolutionarily conserved secreted proteins that provide critical functions during development. Although Wnt proteins share highly conserved features, they also show sequence divergence, which almost certainly contributes to the variety of their signaling activities. We previously reported that *DWnt4* and *wingless* (*wg*), two divergent clustered *Wnt* genes, can have either antagonist or distinct functions during *Drosophila* embryogenesis. Here we provide evidence that both genes can elicit similar cellular responses during imaginal development. Ectopic expression of DWnt4 along the anterior/posterior (A/P) boundary of imaginal discs alters morphogenesis of adult appendages. In the wing disc, DWnt4 phenocopies ectopic Wg activity by inducing notum to wing transformation, suggesting similar signaling capabilities of both molecules. In support of this, we demonstrate that DWnt4 can rescue *wg* loss-of-function phenotypes in the antenna and haltere and is able to substitute for Wg in wing field specification. We also show that both genes are transcribed in overlapping domains in imaginal discs, suggesting that *DWnt4* may cooperate with *wg* during limb patterning.**

Key Words: **axial patterning; *Drosophila* imaginal disc; *DWnt4*; *wg*; *Wnt* signaling.**

INTRODUCTION

Wnt genes play fundamental roles in cellular proliferation, establishment of morphogenetic fields, and cell fate determination (Cadigan and Nusse, 1997; Miller *et al.*, 1999). The encoded proteins form a large family of secreted signaling molecules that share structural features but also show considerable sequence diversity. How this diversity contributes to the wide range of biological functions of Wnt proteins is not precisely understood.

The study of *wg*, identified by virtue of its requirement during wing development, and of its vertebrate homolog Wnt1, led to the characterization of a highly conserved, canonical Wnt signal transduction cascade (Hsieh *et al.*, 1999b; Perrimon, 1995). A variety of regulatory mechanisms modulating the effects of Wg/Wnt proteins are operating at many steps in the pathway. Secretion from producing cells appears to be a controlled process, which in *Drosophila* involves the product of the *porcupine* gene (Kadowaki *et al.*, 1996). Similarly, glycoaminoglycans are required for the presentation of Wg/Wnt ligands at the cell surface (Lin and Perrimon, 1999; Tsuda *et al.*, 1999), while distinct classes of secreted molecules (Glinka *et al.*, 1998; Hsieh *et al.*, 1999a; Piccolo *et al.*, 1999; Rattner *et al.*, 1997) counteract signaling by interfering with reception by Frizzled (Fz)-like proteins (Bhanot *et al.*, 1996; Wang *et al.*, 1996). Through several cytoplasmic relay components including Disheveled, Axin, and GSK-3, the signal is transduced to β-catenin, which then enters the nucleus and forms a complex with TCF to activate Wnt transcriptional targets (Nusse, 1997).

The extent to which the canonical pathway is used by Wnt proteins is not clear. Although certain Wnt ligands bind a similar set of Fz proteins, others bind distinct Fzs and use divergent downstream signaling components. Because

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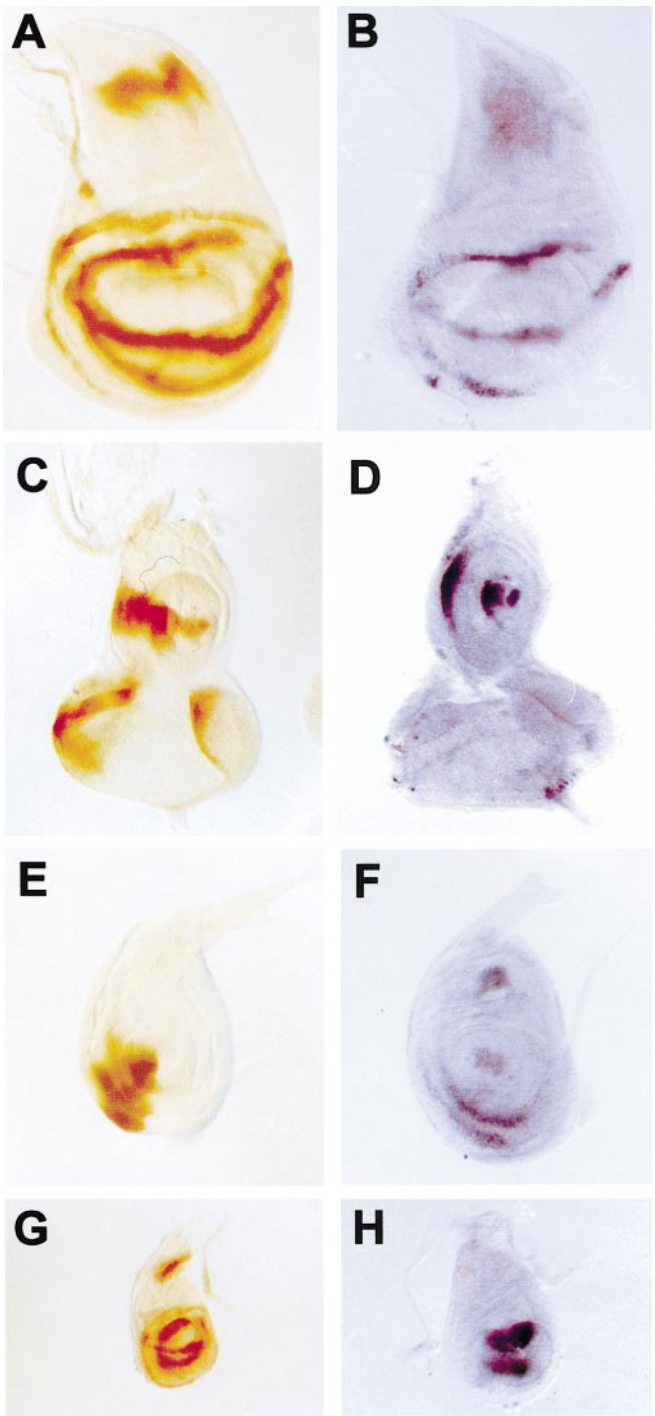


FIG. 1. Expression patterns of *wg* and *DWnt4* in third instar imaginal discs. *wg* expression (A, C, E, and G), visualized by an enhancer trap line (Kassis *et al.*, 1992), was compared to *DWnt4* mRNA distribution (B, D, F, and H) in wild type third instar imaginal discs. In the wing disc, both *wg* (A) and *DWnt4* (B) are expressed at the margin and in the presumptive hinge region. In the notum, where a large *wg* stripe is seen (A), *DWnt4* expression is diffused and weak compared to its expression in the wing blade (B). In the antennal disc, *wg* is transcribed in an anterior–ventral sector (C), and *DWnt4* in a subset only of the *wg*-expressing cells (D). In contrast to *wg*, *DWnt4* is not transcribed in the eye disc. In the leg disc, *wg* expression is ventral in all structures along the proximal–distal axis (E). In this ventral sector, *DWnt4* RNA distribution (F) is restricted to parts of the future leg. Additionally, clusters of proximally located cells that presumably correspond to the sternopleura express *DWnt4*. In the haltere disc, the *wg* pattern is very similar to that observed in the wing disc (G). Similarly, *DWnt4* is transcribed in two large stripes, which may correspond to the pedicel and/or the scabellum. However, *DWnt4* is not expressed in the notum and in the most distal part of the disc (H).

each ligand may be capable of interacting with multiple receptors, whether the canonical Wnt signal transduction machinery is used may depend on the receiving cell type. For example, Wnt5a binding to Fz-2 alters the level of intracellular calcium, presumably through a G-protein-mediated mechanism (Slusarski *et al.*, 1997), while its reception by Fz-5 stimulates the canonical Wnt pathway (He *et al.*, 1997). Similarly, the specification of dorsal cell fates by Wnt7a in the chick limb is apparently not mediated by β-catenin (Kengaku *et al.*, 1998), but the canonical Wg/Wnt pathway does appear to be involved in Wnt7a signaling in the brain (Hall *et al.*, 2000). These data suggest that the activity of a Wnt protein may depend on the Fz molecule that is available. Reciprocally, individual Fz receptors may also "choose" their downstream cascade. Thus, *Drosophila* Fz can initiate either the canonical Wnt pathway or the Planar Cell Polarity pathway (Strutt *et al.*, 1997; Boutros *et al.*, 1998, 2000). It was recently shown that the pathway specificity by the bifunctional receptor Fz is determined by affinity for Wg (Rulifson *et al.*, 2000).

In *Drosophila*, six other *Wnt* genes (*DWnt2, 3/5, 4, 6, 8,* and *10*) besides *wg* have been identified (Adams *et al.*, 2000; Eisenberg *et al.*, 1992; Graba *et al.*, 1995; Russell *et al.*, 1992). Mutants have been described for only one, *DWnt2*, which specifies a sexually dimorphic cell fate during the development of the male reproductive tract (Kozopas *et al.*, 1998). Remarkably, four *Wnt* genes, *wg, DWnt4,* and the recently identified but not yet characterized *DWnt6* and *DWnt10*, are clustered at the 28A cytogenetical position. *DWnt4* and *wg* show closely related embryonic expression patterns. The two genes are significantly divergent in terms of coding sequence and of intron/exon structure. Strikingly, DWnt4 displays an unusual C-terminus marked by two deletions between cysteines 13/14 and cysteines 14/15 (Graba *et al.*, 1995). These data therefore suggest that *wg* and *DWnt4* have been maintained in a close physical linkage during evolution because of shared *cis*-regulatory elements and that this feature may be indicative of related functions during development.

Several examples of clustered "sister genes" exist, including *knirps* and *knirps related* (Rothe *et al.*, 1989), *goosberry proximal* and *distal* (Baumgartner *et al.*, 1987), *sloppy*

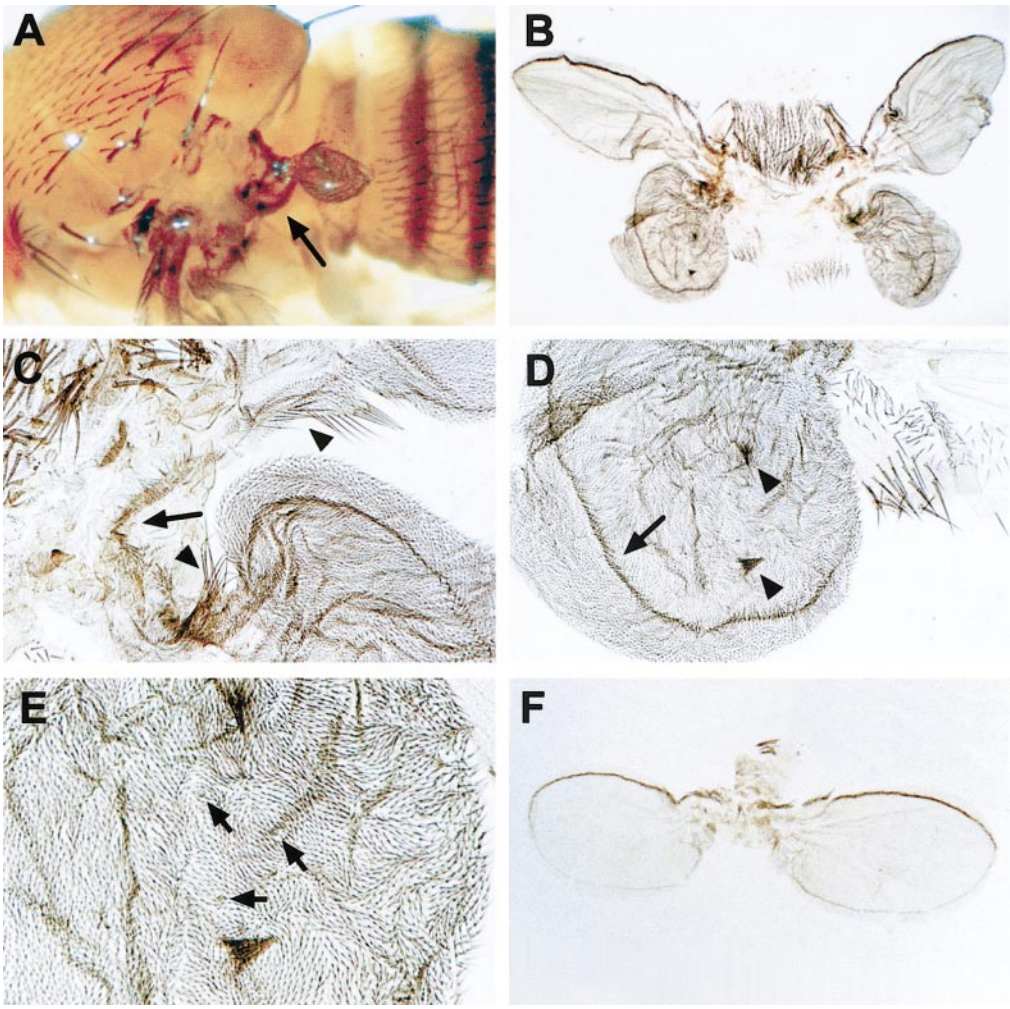


FIG. 2. Ectopic *DWnt4* induces wing duplication. One (A–E) or two (F) copies of UAS*DWnt4* were driven with *ptc*Gal4. (A) In animals raised at 29°C until mid-third larval instar and then shifted to 18°C, small ectopic wings arise from the posterior region of the notum near the boundary between dorsal and ventral notal structures. Note that parts of the scutellum are lost. (B) Dorsal view of thorax and wings of a pharate adult raised at 29°C throughout development. Two large ectopic wings are formed which arise from both sites of the posterior notum. (C–E) Higher magnifications from (B). The ectopic wing is a mirror duplication of the endogenous wing, as illustrated by the relative orientation of allular lobes (arrowheads in C) and a common axillary cord (arrow in C). Ectopic wings have bristle margin of posterior identity (arrow in D). Anterior structures are occasionally formed as indicated by the presence in the ectopic wing blade of anterior margin bristles (arrowheads in D) and campaniform sensory neurons (arrows in E). (F) Complete wing duplication is occasionally observed when two copies of UAS*DWnt4* are expressed under the control of *ptc*Gal4 at 29°C. The ectopic wing show well-formed anterior and posterior compartments and wing margin. This phenotype is associated with loss of nearly the entire notum (not shown).

paired 1 and 2 (Grossniklaus *et al.*, 1992), *zerknult* 1 and 2 (Rushlow *et al.*, 1987), and *engrailed* (*en*) and *invected* (*inv*) (Coleman *et al.*, 1987). Ectopic expression experiments revealed that the homologous gene products have the potential of inducing similar developmental defects (Cadigan *et al.*, 1994; González-Gaitán *et al.*, 1994; Li and Noll, 1994). Some of these gene pairs exhibit distinct expression patterns, which exclude redundant functions during development. This is the case for the *gooseberry* genes, in that one is expressed and functions in the epidermis and the other in the central nervous system (Baumgartner *et al.*, 1987). For other gene pairs, identical expression profiles suggest either functional redundancy or specialized functions in the same developmental processes. This is best illustrated by the *en*/*inv* pair, because only *en* mutation results in embryonic lethality, while ubiquitous expression of either gene causes embryonic segments to fuse (Gustavson *et al.*, 1996; Simmonds *et al.*, 1995; Whiteley and Kassis, 1997). This indicates that, although the two genes have the same expression pattern, Inv cannot substitute for

En during embryonic development. Mutations in *inv* are viable, but careful examination of adult phenotypes of *en* single mutants or *inv en* double mutants allowed specific functions to be assigned to each gene (Simmonds *et al.*, 1995).

We previously investigated the functional relationship of *DWnt4* and *wg* during embryonic development and found they have antagonistic or distinct activities in ventral (Gieseler *et al.*, 1999) or dorsal parts of the epidermis (Buratovich *et al.*, 2000), respectively. Here we report an analysis of *DWnt4* activity during imaginal development. As during embryogenesis, *wg* and *DWnt4* expression profiles are similar but not identical. Noticeably, we found that *DWnt4* is transcribed at the dorsoventral (D/V) compartment boundary of the third instar wing disc. When ectopically provided at the A/P compartment boundary using the UAS/Gal4 system, DWnt4 profoundly affects adult appendage formation. Some of the induced phenotypes resemble that caused by ectopic Wg. In addition, we demonstrate that DWnt4 can substitute for several Wg functions during antenna, haltere, and wing morphogenesis. These results indicate that, despite important sequence divergence, the two proteins can elicit similar cellular responses during imaginal development.

MATERIALS AND METHODS

Fly Strains, Fly Rearing, and Ectopic Expression of DWnt4

Oregon R was used as wild type standard. Mutant stocks were obtained from the Bloomington Centre. The construction of UAS-*DWnt4* transgene and generation of transformed lines are described elsewhere (Gieseler *et al.*, 1999). The UAS/Gal4 system was used to ectopically express *DWnt4,* using the *patched* (*ptc*)Gal4 driver. Pharate adults were dissected from pupae in Ringer's solution, mounted in 90% glycerol, and observed under an AxioPhot Zeiss microscope using Nomarski optic.

In Situ Hybridization and Immunostaining on Discs

Discs were dissected from third instar larvae in Ringer's solution, fixed, and immunostained as described in Johnston and Schubiger (1996). Primary antibodies against Cubitus interruptus (Ci), En, and Vestigial (Vg) were kindly provided by R. Holmgren, A. Vincent, and S. Carroll. Secondary antibodies, FITC or TRITC labeled or biotinylated, were from Jackson ImmunoResearch Laboratories (West Grove, PA). Confocal microscopy was performed on a Zeiss confocal microscope and data were processed with Adobe Photoshop. *In situ* hybridizations were performed using digoxigenin DNA-labeled probes (Boehringer), using a full-length *DWnt4* cDNA or a 1-kb fragment lying in the 5′ coding sequence of the *wg* gene. This fragment was obtained from genomic DNA by PCR amplification using the following primers: 5′-AAGTTGG-CGCCCTTGACCAG and 5′-GCCGTGTGATCCAGCGGAAT. After alkaline phosphatase detection, the discs were mounted in 90% glycerol, 100 mM Tris, pH 7.5, and observed under an AxioPhot Zeiss microscope using Nomarski optic.

RESULTS

DWnt4 Expression in Third Instar Imaginal Discs

wg and *DWnt4* are transcribed in overlapping patterns during embryogenesis (Gieseler *et al.*, 1995; Graba *et al.*, 1995). They also show largely similar expression profiles in third instar imaginal discs (Fig. 1). In the wing disc, transcripts of the two genes are synthesized at the D/V compartment border, the future wing margin, and the presumptive wing hinge region (Fig. 1A). *DWnt4* expression, however, is weak compared to that of *wg* and appears more spatially restricted. It proceeds following a cell stripe comparable in width to the *wg* stripe in the distal part of the future wing margin, but is more faintly detected in more proximal parts of anterior and posterior compartments. The patterns are different in the presumptive notum, where *wg* RNA is found in a broad D/V stripe, whereas *DWnt4* transcripts faintly label a central cell domain (Fig. 1B). These cells are located beneath the columnar epithelium and, therefore, likely correspond to adepithelial cells. In antenna and leg discs, the *wg* domain corresponds to a ventral/anterior sector (Figs. 1C and 1E) (Baker, 1988b), whereas *DWnt4* is expressed only in a subset of these cells (Figs. 1D and 1F). Whereas *wg* RNA is detected along the entire proximal–distal axis in the leg disc, *DWnt4* transcription is restricted to two segments which correspond to primordia of the tibia and a more distal segment and in a dorsally located cell cluster that presumably corresponds to the sternopleural region. The *wg* pattern in the haltere disc is reminiscent of that observed in the wing disc (Fig. 1G). *DWnt4* is transcribed in two large stripes, which will give rise to the pedicel and/or the scabellum. However, in contrast to *wg, DWnt4* is not expressed in the notum and in the most distal part of the disc, which is homologous to the margin of the wing (Fig. 1H). A clear difference in expression of the two genes is also seen in the larval central nervous system, where *wg* is not expressed (Baker, 1988a,b), while *DWnt4* transcripts follow a segmentally repeated pattern of small clusters in the central cortex and in the optic anlagen (not shown).

In summary, the transcription profile of *DWnt4* in third instar imaginal discs partially overlaps that of *wg* but is not restricted to *wg*-positive cells. For example, in the wing blade, the two genes are expressed along the future margin and in the presumptive hinge region, suggesting that the encoded Wnt products may be required together for the differentiation of these structures. In the central nervous system or in the dorsal part of the leg disc, *DWnt4* is expressed where *wg* is not, suggesting it may also function in cells that do not depend on *wg.*

Ectopic DWnt4 Induces Wing and Haltere Duplications

To assess the ability of *DWnt4* to affect axial patterning, we used the *ptc*Gal4 driver, which promotes expression at

the A/P compartment boundaries of imaginal discs, in combination with chromosomes bearing one or two copies of a UAS*DWnt4* transgene. One copy of *DWnt4* driven by *ptc*Gal4 at 18°C leads to adult flies with outspread wings (not shown). When embryos are reared at 29°C to increase Gal4 activity and are shifted back to 18°C at third instar, most flies die as pharate adults. However, some escapers hatch that occasionally show small ectopic wings arising at the D/V boundary in the posterior region of the notum (Fig. 2A). Raising the flies continuously at 29°C induces stronger phenotypes; about 20% of the progeny reach the pharate adult stage and develop ectopic wings of different sizes. While most of them remain small and exhibit hairlike structures characteristic of the allula (not shown), large ectopic wings are reproducibly obtained (Fig. 2B). The supernumerary wing develops as a mirror duplication of the endogenous one. This is illustrated by the mirror image of the allular lobes (arrowheads in Fig. 2C) and by the existence of a common axillary cord connecting the ectopic and endogenous wings (arrow in Fig. 2C). The supernumerary wing is usually surrounded by thin and short bristles characteristic of the posterior margin (arrow in Fig. 2D). However, anterior structures are found, as illustrated by the presence at abnormal position of large sensory bristles characteristic of the anterior wing margin (arrowheads in Fig. 2D) and by the formation of campaniform sensory neurons in the ectopic wing blade (arrows in Fig. 2E). Of note, the wing duplication phenotype is associated with a strong reduction of the scutellum (Fig. 2B), suggesting that the extra wing forms at the expense of scutellar cells.

We further raised the levels of *DWnt4* expression by using two copies of the UAS*DWnt4* transgene at 29°C. Under these conditions, large ectopic wings are found at higher frequency and, in rare cases (1% of dissected pharates), fully duplicated wings arise with well-formed anterior and posterior compartments and margin (Fig. 2F). These flies also lack nearly the entire notum (not shown). Taken together these results indicate that DWnt4 provided from the A/P compartment boundary induces cells normally contributing to notum formation to take on a wing fate. This effect, notum to wing transformation, occurs in a dose-dependent manner, because low levels of DWnt4 induce small ectopic wings and partial reduction of scutellar and notal structures, whereas higher levels eventually cause complete wing duplication and loss of the entire notum.

UAS*DWnt4* driven by *ptc*Gal4 at 29°C also reproducibly induces duplications of halteres. The bifurcation occurs more or less distally, in that the two resulting appendages either are completely separated, form a common scabellum, or have fused pedicels (Fig. 3). These phenotypes apparently do not depend on the DWnt4 dose, because one or two copies of the transgene similarly produce partial or complete duplications.

DWnt4 Reorganizes Pattern Formation in the Wing Disc

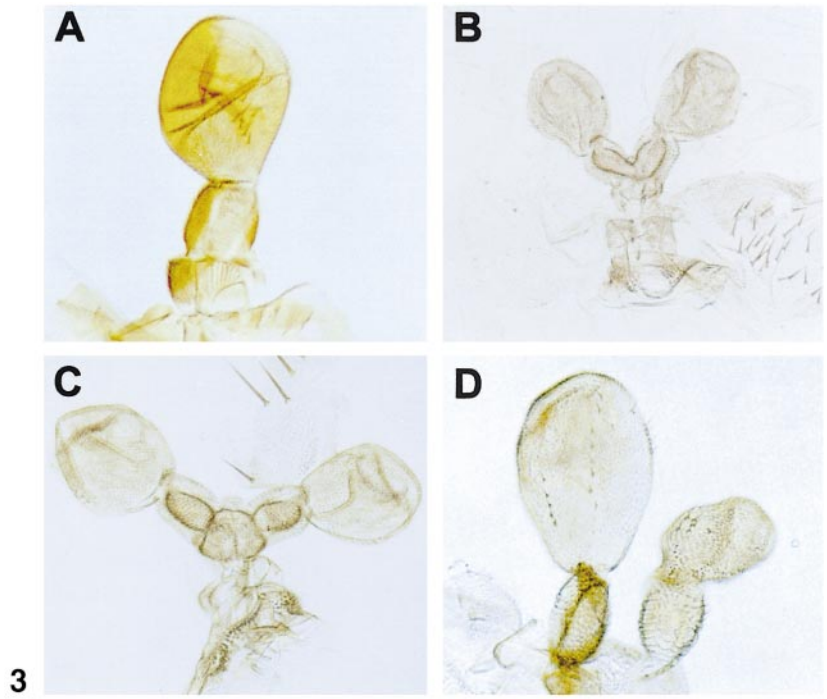
To investigate the basis of supernumerary wing formation induced by DWnt4, the expression of four wing-patterning genes, *en*, *ci*, *vg*, and *wg* as well as of *DWnt4* itself, was monitored in *ptc*Gal4-UAS*DWnt4* imaginal discs. Wing discs from this genotype show outgrowth from the posterior region of the notum, outgrowth that corresponds to the future ectopic wing blade (Fig. 4).

In the wild type wing disc, En is found in all cells of the posterior compartment (Kornberg *et al.*, 1985) (Fig. 4A) and Ci, a transcriptional effector of the Hedgehog pathway, is specifically produced in the anterior compartment with higher levels in a band of cells abutting the A/P boundary (Orenic *et al.*, 1990) (Fig. 4C, green). This boundary is barely distinguishable in the outgrowth of *ptc*Gal4-UAS*DWnt4* discs, where En is expressed in a large domain that overlaps most of the ectopic wing field (Fig. 4B) and Ci rather accumulates in comparatively fewer cells (arrow in Fig. 4D).

The *vg* gene is activated by organizing signals from both A/P and D/V boundaries (Klein and Martinez-Arias, 1999; Nagaraj *et al.*, 1999) and is expressed throughout the wing blade and in cells that extend beyond the D/V border in the posterior and anterior compartments (Fig. 4C, red). Vg function is essential for wing development and is able to promote the formation of winglike structures at ectopic positions (Kim *et al.*, 1996). In the ectopic wing field induced by DWnt4, the Vg protein is expressed at high levels (Fig. 4D). In addition, *wg* transcripts are found in the supernumerary and endogenous D/V borders (Fig. 4F). Consistent with the posterior identity of margin bristles, the ectopic wing blade, which is delineated by Vg and *wg* expressions, mostly falls in the En domain. However, some cells in this domain produce the Ci effector and are therefore fated to express an anterior phenotype (arrow in Fig. 4D). This is consistent with the sporadic formation of campaniform sensory neurons and anterior margin bristles in pharate adult ectopic wings. Finally, *DWnt4* transcription, besides induction by *ptc*Gal4 at the A/P border, is strongly activated along the D/V axis of the future ectopic wing blade (arrow in Fig. 4H). This expression at the ectopic margin is best understood as resulting from induction by ectopic Wg, consistent with the well-known capability of this protein to induce a whole new margin to form, along with all the margin genes examined (Grimm and Pflugfelder, 1996; Ng *et al.*, 1996).

Ectopic DWnt4 Induces Supernumerary Bristles

In addition to effects on the axial pattern of haltere and wing, ectopic DWnt4 produces supernumerary bristles in specific regions. First, DWnt4, like Wg (Neumann and Cohen, 1996), induces extra sternopleural bristles (Fig. 5B). This may reflect an endogenous activity of DWnt4, in that, unlike *wg*, it is transcribed in a dorsal domain of the leg disc that roughly corresponds to the sternopleura (see Fig. 1).



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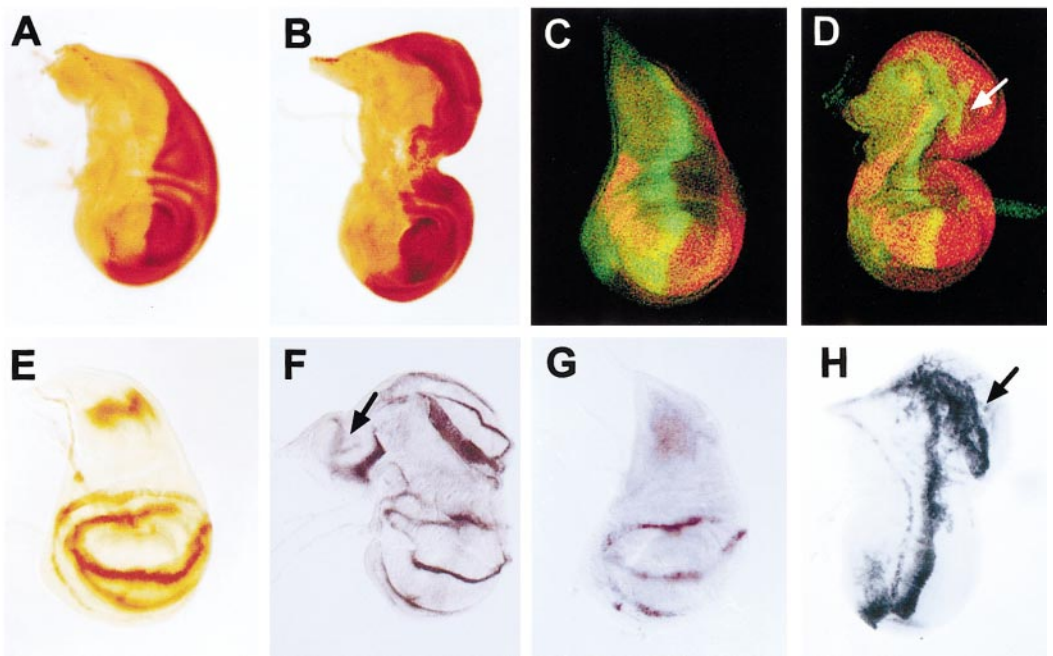

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FIG. 3. Ectopic *DWnt4* expression induces axis duplications in the haltere. Haltere defects resulting from one copy of UAS*DWnt4* expressed under the control of *ptc*Gal4 at 29°C. (A) Wild type control. (B–D) Increasing severity in the appendage mutant phenotype. Note that bifurcation occurs at different positions along the proximal–distal axis.

FIG. 4. Expression of wing-patterning genes induced by ectopic *DWnt4* expression in wing imaginal discs. Wing imaginal discs, prepared from third instar larvae raised at 29°C and bearing two copies of UAS*DWnt4* driven by the *ptc*Gal4, were stained for En, Ci, and Vg proteins and for *wg* mRNA. Note the abnormal morphology provoked by outgrowing tissue from the posterior region of the notum, which corresponds to the future ectopic wing blade (B, D, F, and H). (A) Wild type pattern of the En protein (brown) in the posterior compartment. (B) En is expressed in the posterior part of the ectopic wing blade. (C) Wild type expression of Ci (green) and Vg (red) proteins. Ci marks all anterior cells and is highly expressed at the A/P boundary, while Vg is detected at the D/V boundary and in surrounding cells from posterior and anterior compartments (D). In the ectopic wing blade only few cells accumulate Ci (arrow), whereas Vg is highly induced in the ectopic wing pouch. (E) β-Gal staining reproducing the wild type *wg* pattern. (F) *wg* expression in the ectopic wing pouch is similar to that observed in the endogenous. Note the duplicated stripe of *wg* expression in the notum (arrow). (G) Wild type expression of *DWnt4*. (H) In the ectopic wing pouch, *DWnt4* is highly induced along the D/V axis (arrow). Note the strong activation of UAS*DWnt4* by *ptc*Gal4 at the A/P border of the disc. In comparison, transcription of the endogenous gene, which occurs by far at a lower rate, is barely distinguishable.

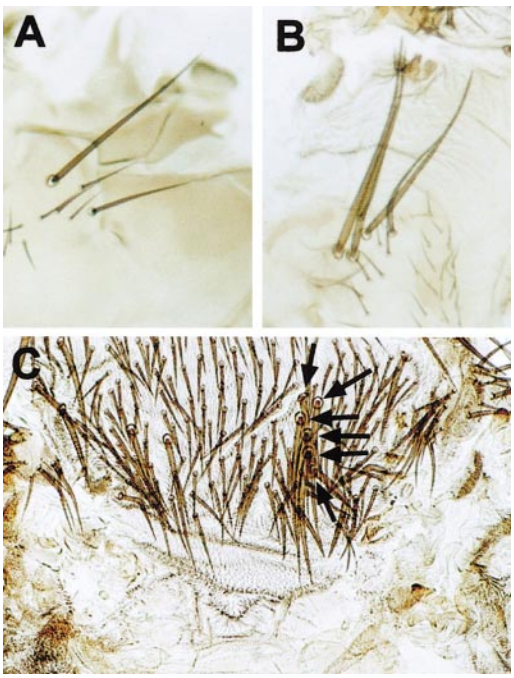

FIG. 5. Ectopic *DWnt4* induces bristle duplications. (A) Wild type sternopleural bristles. The number of these bristles is somewhat variable, but generally, two long bristles, one midsized bristle, and a cluster of five to seven small bristles are present. (B) The sternopleural region of a *ptc*Gal4-UAS*DWnt4* adult (two copies of the transgene) raised at 29°C. Three midsized bristles and two long bristles are present. The small bristles are out of the plane of focus. (C) Notum of an animal bearing two copies of UAS*DWnt4* driven by *ptc*Gal4 at 29°C. Note the loss of the scutellum and dorsocentral bristle duplications (arrows).

Second, DWnt4 produces supernumerary dorsocentral bristles (Fig. 5C), posterior postalar bristles, and scutellar bristles, when the scutellum is not transformed into wing (not shown). Precursors of these bristles are specified by *wg* (Phillips and Whittle, 1993) and proximal expansion of *wg* expression in the notum leads to ectopic dorsocentral bristles (Calleja *et al.*, 1996). In *ptc*Gal4-UAS*DWnt4* wing discs, an additional stripe of *wg* expression appears in the proximal notum (arrow in Fig. 4F), suggesting that the excess of notal bristles in flies of this genotype is most likely the result of ectopic Wg activity in the notum.

Wg activity is also required for D/V and proximodistal pattern in the leg and antenna (Baker, 1988a), for specification of wing margin bristles (Phillips and Whittle, 1993), and for head patterning (Royet and Finkelstein, 1996, 1997). In addition, ectopic Wg inhibits the formation of sensory organ precursors in the eye (Cadigan and Nusse, 1996). Interestingly, ectopic DWnt4 cannot impinge on these processes in our analyses, indicating that it is unable to elicit responses in many Wg-responsive cells.

DWnt4 Rescues Phenotypes Associated with Wingless Loss of Function

The previous results together show that *DWnt4* has the capacity to influence several *wg*-dependent processes. This

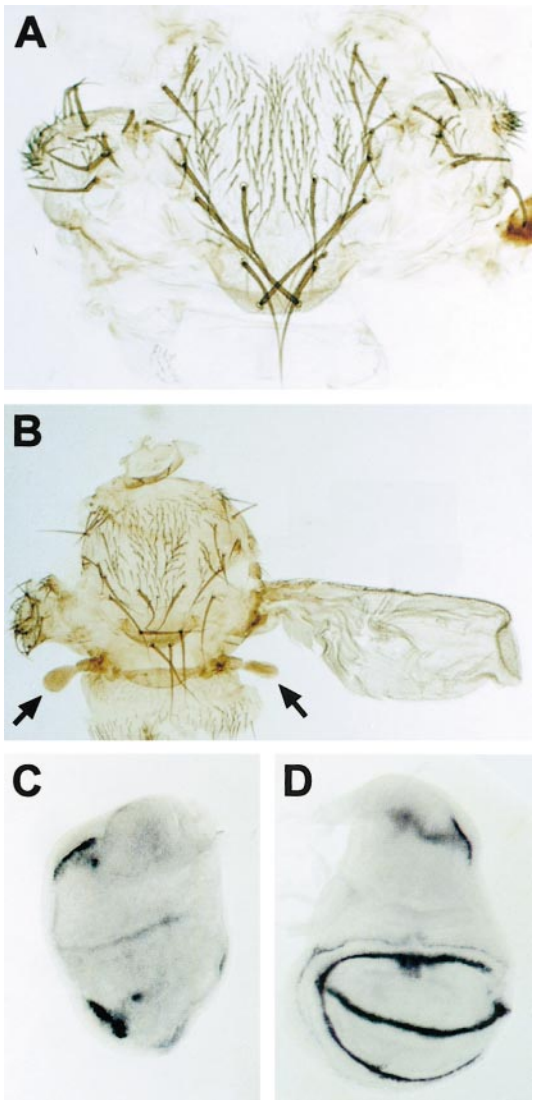

FIG. 6. *DWnt4* rescues wing and haltere phenotypes of wg^{CX3}/wg^{CX4} mutants. (A) Dorsal view of the thorax of a wg^{CX3}/wg^{CX4} mutant pharate adult. Wings on both sides are transformed into notum; note in addition the absence of halteres. (B) Dorsal view of the thorax of a wg^{CX3}/wg^{CX4}; *ptc*Gal4-UAS*DWnt4* (two copies of the transgene) pharate adult raised at 29°C. Normal wing formation has been restored on the right side, while the left wing still is transformed into notum. Haltere formation is restored on both sides (arrows). (C, D) Two wing imaginal discs from the same *ptc*Gal4-UAS*DWnt4*;wg^{CX3}/wg^{CX4} third instar larva. (C) The wing blade is replaced by a mirror duplication of the notum, which is typical for wg^{CX3}/wg^{CX4} mutants. *wg* expression is observed in the normal and the duplicated notum. (D) The second wing imaginal disc prepared from the same animal has a normal morphology and presents a wild type *wg* expression.

suggests that it either mimics *wg* activity, acts as a mediator of Wg signaling, or functions through the induction of *wg* expression. To address these possibilities, we tested the effect of *DWnt4* in mutant backgrounds deficient for *wg* during imaginal development.

We first used the heteroallelic combination wg^{CX4}/wg^{CX3}, which leads to pharate adults with wing to notum transformations and loss of halteres (Baker, 1988a) (Fig. 6A). Whereas wg^{CX4} is an amorphic allele, the wg^{CX3} chromosome contains a deficiency in the 3′ untranscribed region of the gene (Baker, 1988a) but has conserved the 5′ enhancer element that drives expression in the wing hinge and margin in the third larval instar (Neumann and Cohen, 1996). wg^{CX3} lacks the *wg* function required for wing field establishment at the second larval instar (Klein and Martinez-Arias, 1998). In a wg^{CX4}/wg^{CX3} background, *ptc*Gal4-driven ectopic *DWnt4* is able to rescue most of the described phenotypes. We observed wing rescue at high frequency, often leading to pharate adults bearing a normal wing on one side and a wing to notum transformation on the other (Fig. 6B). Halteres quasi-systematically appear well developed (Fig. 6B, arrows). This indicates that ectopic *DWnt4* can complement wg^{CX3} loss of function. Further, we examined *wg* expression in wg^{CX4}/wg^{CX3} wing imaginal discs rescued by *ptc*Gal4-UAS*DWnt4* and found that a normal pattern of *wg* expression is restored in the rescued disc (Figs. 6C and 6D). Because the pattern of *wg* at the third instar depends on earlier *wg* function, this indicates that DWnt4 can substitute for Wg during wing field specification.

wg^{CX4}/wg^{CX3} pharate adults exhibit additional phenotypes, including loss of antennae and dorsalization of ventral leg structures (Figs. 7C and 7D). Legs from *ptc*Gal4-UAS*DWnt4* animals very often recover a normal morphology (Fig. 7E). The antenna is less susceptible to phenotypic correction by DWnt4, because only partial rescue was occasionally obtained (Fig. 7F). These results indicate that DWnt4 can substitute for Wg activity not only during wing field specification, but also during leg and antenna development.

In a second set of experiments, *DWnt4* expression was induced by *ptc*Gal4 in the *wg* temperature-sensitive heteroallelic combination wg^{CX4}/wg^{IL114}. Upshift from permissive (18°C) to restrictive (29°C) temperature results in the removal of *wg* function and in the concomitant increase of Gal4-driven expression of *DWnt4*. Wing discs from this genotype, shifted to 29°C for 24 h at the beginning of the second larval instar and then transferred back to 18°C before dissection in the late third instar, show normal morphology with fully developed wing pouch and a wild type pattern of Vg expression (Fig. 8B). In contrast, discs raised in the same conditions, but missing the UAS*DWnt4* transgene, consistently exhibit pronounced wing to notum transformation and erratic Vg expression (Fig. 8A). These observations confirm that, early during the second larval instar, *DWnt4* has the ability to supply a Wg-like activity sufficient for wing field specification. When Wg activity is removed from the second instar, by continuously maintaining larvae at the restrictive temperature until late third instar, imaginal discs fail to develop normally and remain very small without Vg expression (Fig. 8C). Thus, *DWnt4* driven by *ptc*Gal4 cannot supply the subsequent *wg* activities necessary for wing growth and patterning.

DISCUSSION

In this study we first examined the *DWnt4* transcription pattern compared to that of *wg* in third instar imaginal discs. The data revealed partially overlapping expression patterns, indicating that some cells are exposed to both signaling molecules, while others most probably respond only to either DWnt4 or Wg. We further addressed the patterning capability of DWnt4 by ectopically expressing it at the A/P compartment boundary. DWnt4 was found to induce severe developmental abnormalities both in the specification of a complete cellular field and in the specification of single cell fates. Some of the defects are phenocopies of alterations caused by ectopic Wg, supporting the conclusion that, despite significant sequence divergence, both molecules can elicit similar responses. This is further demonstrated by the ability of DWnt4 to rescue *wg* loss-of-function phenotypes.

Ectopic DWnt4 Induces Distinct Classes of Phenotypes and Mimics Ectopic Wg Activity

Wing duplication can originate from at least two distinct processes. One results from perturbation in the morphogenetic pattern after the wing field has been established. The best illustrations are those obtained from misregulation of the D/V patterning gene *apterous* (Diaz-Benjumea and Cohen, 1993), as well as from ectopic expression of A/P factors like En, Hedgehog, or Decapentaplegic (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata *et al.*, 1995). In these cases, the additional wing originates from an axial duplication of cells normally fated to form the wing and the duplicates always share with the endogenous appendage the attachment to the body wall. A second process results from defects in early wing field establishment. Specification of wing versus notum occurs during the second and early third instars. It relies on the spatially restricted expression of *wg* and its partial overlap with that of *vg* (Klein and Martinez-Arias, 1998). Accordingly, ectopic activities of Wg or of Wg downstream effectors induce supernumerary wings (Couso *et al.*, 1995; Grimm and Pflugfelder, 1996; Ng *et al.*, 1996) that do not emanate from a common body wall attachment, but form at the expense of hinge and/or notal structures.

An important feature of the duplication induced by DWnt4 is that ectopic and endogenous wings are always separated from one another. The extra wing originates from the posterior notum near the boundary between dorsal and ventral structures and is clearly formed at the expense of notum and scutellum. This indicates that the duplication process results from a respecification of cells normally fated

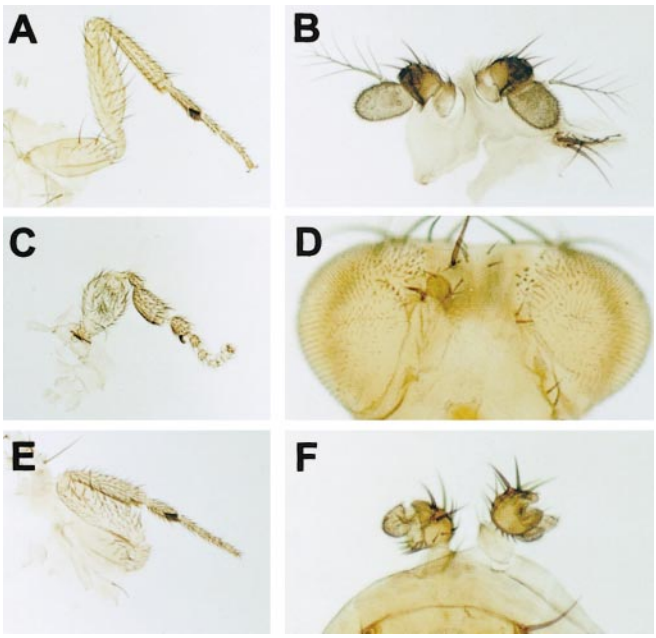

FIG. 7. *DWnt4* rescues leg and antenna phenotypes of wg^{CX3}/wg^{CX4} mutant. (A) First leg of a wild type male. (B) Wild type antennae. In wg^{CX3}/wg^{CX4} mutant flies, leg structures are dorsalized (C) and the antennae are nearly completely lost (D). *DWnt4* expression (two copies of the transgene) driven by *ptc*Gal4 at 29°C in a wg^{CX3}/wg^{CX4} background, often rescues the leg phenotype (E), while the formation of antennae is only partially restored (F).

to become body wall elements. Reprogramming of notum cells by DWnt4 is consistently illustrated by the ectopic induction of wing-patterning genes. Additional sites of *en*, *ci*, and *wg* expression in the posterior outgrowth of mutant discs indicate that new compartments and boundaries are formed and are able to provide positional information required for *vg* transcription and development of an ectopic wing pouch. Even more demonstrative of a complete reprogramming of notal cells is the appearance, in rare instances, of fully developed and patterned wings.

The phenotypes seen in the haltere most certainly also result from respecification of a complete cellular field. However, there are obvious differences in the effects of ectopic DWnt4 on haltere and wing development. Even one copy of UAS*DWnt4* induces the formation of separated wings, and the levels only define the completeness of the notum to wing transformation. In contrast, fully separated halteres or partial duplications branching at different places along the haltere proximal–distal axis apparently result from the same level of ectopic DWnt4 expression. This suggests that ectopic DWnt4 initiates distinct mechanisms in the two appendages and/or that many cells along the proximal–distal axis of the haltere disc are competent to respond to DWnt4.

Finally, the supernumerary notal and sternopleural bristles that are specified in *ptc*Gal4-UAS*DWnt4* animals suggest that DWnt4 is also able to influence individual cell fate. Distinct mechanisms may account for this activity. In the wing disc, the development of supernumerary bristles is associated with the activation of *wg* in a second stripe in the notum. Because Wg is required for the specification of notal bristles and can also cause ectopic bristle formation (Phillips and Whittle, 1993), DWnt4 presumably induces ectopic bristles indirectly through activation of Wg. The situation might be different in the leg disc. First, *DWnt4*, but not *wg*, is expressed in the same region of the leg disc that produces sternopleural bristles, suggesting an endoge-

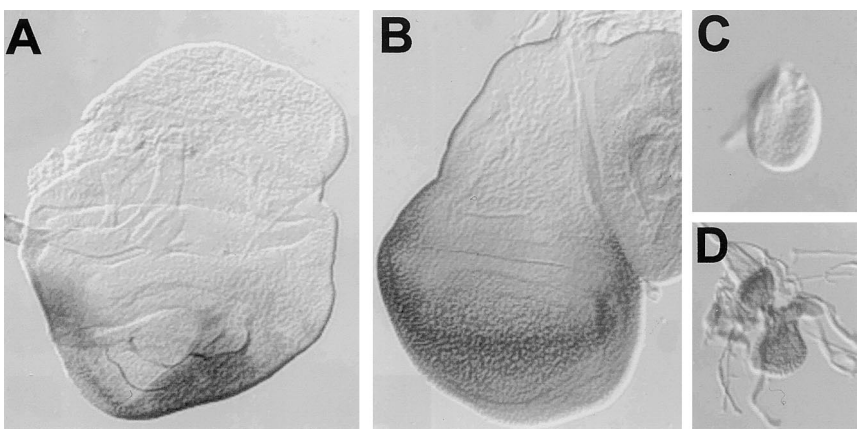

FIG. 8. *DWnt4* can substitute for *wg* in wing field specification. All discs were stained for the Vg protein. (A, B) Wing discs from third instar wg^{CX4}/wg^{IL114} larvae, grown at 18°C for 60–70 h AEL and shifted to 29°C for 24 h, in the absence (A) or presence (B) of *ptc*Gal4 and one copy of the UAS*DWnt4* transgene. Note that DWnt4 restores the formation of the wing pouch and normal Vg expression. (C, D) Wing discs from third instar wg^{CX4}/wg^{IL114} larvae, grown at 18°C for 60–70 h AEL and shifted to 29°C, in the absence (C) or presence (D) of *ptc*Gal4 and one copy of the UAS*DWnt4* transgene. Note that discs fail to develop, even when *DWnt4* is provided.

nous activity of DWnt4 in this process. Second, the *Sternopleural* mutation induces the formation of extra sternopleural bristles without ectopic *wg* activation in the appropriate region of the disc (Buratovich *et al.,* 1997; Neumann and Cohen, 1996), indicating that Wg is dispensable for the expression of the phenotype. The capability of both Wg and DWnt4 to elicit similar cellular responses (extra sternopleural bristles) suggests that Wg may act indirectly through activation of DWnt4. Alternatively, an uncharacterized (Wnt) protein may be involved in the specification of these bristles, and both DWnt4 and Wg may mimic this activity.

Context Dependence of DWnt4 Activity: DWnt4 Can either Antagonize or Substitute for Wg Activity

The ability of DWnt4 to induce, as Wg does, additional wings indicates that the two molecules can elicit similar cellular responses. Strong support for this conclusion is provided by rescue experiments of *wg* loss-of-function phenotypes. wg^{CX4}/wg^{CX3} animals die as pharate adults and express a variety of defects, such as transformation of wing and haltere in notum and metanotum, absence of antennae, and loss of ventral leg structures (Baker, 1988a,b). All these phenotypes can be fully (wing and haltere) or partially (leg and antennae) rescued by DWnt4. However, since the wg^{CX3} chromosome does not affect the promoter and coding regions, it is formally possible that the rescue by DWnt4 is the result of transcriptional activation of *wg* during wing field specification. This is not the case, because using the wg^{CX4}/wg^{IL114} allelic combination we demonstrated the DWnt4 ability to restore normal wing development in the absence of a functional Wg protein at the second instar.

In the same genetic context, eliminating Wg signaling further during the third instar, allows wing field specification but not subsequent wing patterning and growth. DWnt4 therefore appears unable to substitute for Wg signaling during late wing development. This failure to replace Wg might be the result of ectopic expression in inappropriate domains or of distinct signaling abilities during wing growth and patterning. In support of the latter possibility, the two Wnt molecules produce different effects, depending on the developmental context. Ectopic expression of Wg, but not of DWnt4, perturbs leg, eye, or antenna development, which reveals competency to respond to Wg but not to DWnt4. In addition, previous work showed that DWnt4 is able to antagonize late embryonic Wg signaling in the *Drosophila* ventral epidermis and to block the Wg-induced body axis duplication in *Xenopus* (Gieseler *et al.,* 1999). Furthermore, the two genes induce different phenotypes in the dorsal embryonic epidermis (Buratovich *et al.,* 2000). Taken together these results strongly support a context dependence for DWnt4 activity.

The molecular bases underlying the ability of Wg and DWnt4 to perform antagonistic (Gieseler *et al.,* 1999), distinct (Buratovich *et al.,* 2000), or similar (this study) signaling activities remain to be explored. We previously proposed that the DWnt4/Wg antagonistic relationship in *Drosophila* embryonic ventral ectoderm and in *Xenopus* axis induction assay results from strong sequence divergences in the C-terminal parts of the two proteins. This was supported by reports that C-terminal truncations in Wg and XWnt8 result in proteins with dominant negative or antagonistic functions (Bejsovec and Wieschaus, 1995; Couso and Martinez-Arias, 1994; Hoppler *et al.,* 1996). If this interpretation is correct, domains other than the divergent C-terminal ends in the Wnt proteins would be critical for function in other developmental contexts. Although interactions of the C-terminus with specific partners dictate activity in the ventral embryonic epidermis, the recruitment of imaginal disc-specific factors by other domains in Wg and DWnt4 would allow the proteins to exhibit similar activities during wing field specification. The existence of separated functional domains in Wnt proteins is also supported by the nature and effect of the wg^{NEI} mutation, where a single amino acid change affects a subset of Wg functions only (Bejsovec and Wieschaus, 1995; Dierick and Bejsovec, 1998).

An alternative model to explain antagonism versus similarity is that DWnt4 acts as a low-activity agonist of Wg. In this model, tissue-specific differences in receptor concentrations and/or differences in receptor-binding affinities would determine whether DWnt4 mimics or antagonizes Wg signaling. If, for example, receptor concentrations are limiting in the ventral epidermis, DWnt4 may act as a competitive inhibitor of Wg for receptor binding but would provide less stimulation of the pathway than would Wg itself, therefore lowering the level of Wg signaling. However, if receptor concentrations are high, DWnt4 may be able to increase signaling by binding receptors without competing with endogenous Wg. If DWnt4 interacts with Wg receptors with lower affinity than that of Wg, the tissue-specific differences in its ability to engage the Wg signaling pathway might be explained. In particular, this may explain the inability of DWnt4 to stimulate the Wg pathway in wild type legs while stimulating the pathway, and rescuing leg defects, in the absence of Wg.

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